Spring 5-7-2021

The Effect of BTS Induced Inactivity on a Zebrafish Model of Duchenne Muscular Dystrophy

Sean Driscoll
University of Maine, sean.driscoll@maine.edu

THE EFFECT OF BTS INDUCED INACTIVITY ON A ZEBRAFISH MODEL OF DUCHENNE MUSCULAR DYSTROPHY

By

Sean Driscoll

B.S. University of Maine, 2019

A THESIS

Submitted in Partial Fulfilment of the

Requirements for the Degree of

Master of Science

(in Zoology)

The Graduate School

The University of Maine

May 2021

Advisory Committee

Dr. Clarissa Henry, Professor of Biological Sciences, Advisor

Dr. Jarod Talbot, Assistant Professor of Developmental Biology

Dr. Benjamin King, Assistant Professor of Bioinformatics

THE EFFECT OF BTS INDUCED INACTIVITY ON A ZEBRAFISH MODEL OF DUCHENNE MUSCULAR DYSTROPHY

By Sean Driscoll

Thesis Advisor: Dr. Clarissa Henry

An Abstract of the Thesis Presented in Partial Fulfillment of the Requirements for the Degree of Master of Science

(in Zoology)

May 2021

Duchenne's Muscular dystrophy (DMD) is a congenital disease of the muscle characterized by muscle atrophy, weakness, and a lower quality of life. Often diagnosed in children, it affects about 1 in every 5,500-7,700 males. A patient diagnosed with DMD is often told to avoid physical activities outside the required amount needed to go on with their day in order to preserve the muscle fibers and integrity. Inactivity in a healthy person leads to decreased muscle mass and increased weakening of the muscle, so we questioned if the effects may be exacerbated in a person diagnosed with DMD having already weakened muscle. In other words, 'does inactivity make disease progression worse?' In this project, we explored the effects of inactivity on a zebrafish model of DMD. We used N-Benzyl-p-toluene sulfonamide (BTS), a myosin heavy chain inhibitor, to induce total inactivity for 72-hours consecutively at disease onset (2 days post fertilization), followed by a 72-hour recovery period out of BTS where normal activity is resumed. This setup mimics activity levels that a DMD patient may experience; periods of inactivity advised by doctors to preserve muscle fibers followed by use of the muscle for an activity such as physical therapy. We then analyzed the effects on muscle structure (via birefringence) and function (via swimming distance and velocity), as well as survival. Based on

previous studies done on inactivity and muscle health, we hypothesized that extended inactivity would have a deleterious effect on the structure and function of the muscle fibers as well as survival. We found that muscle structure and function was not significantly different than control disease progression after extended inactivity. Survival was also not significantly different from the control. The results of this data show us that while extended inactivity did not worsen disease progression as compared to the control, inactivity did not improve muscle structure and function or survival. This data is an important step in understanding the effects of inactivity on disease progression in DMD in hopes to provide better management and therapy towards DMD.

ACKNOWLEGMENTS

I would like to extend a gracious thank you to Clarissa Henry for her continued support and guidance throughout the process of conducting this project, especially with the crazy schedule changes due to pandemic-related events, my seemingly endless intestinal illnesses, teaching, and constant questions no matter how dumb they may have been. Her perseverance through tough times taught me how to balance a love for science, an incredible work ethic, a fantastic sense of humor, and a love for dogs all at one time.

I would also like to thank each and every member of the Henry Lab, past and present (in no particular order of favorites): Claire, Eli, Sarah, Mary, Kodey, Amanda, Ahmed, Elisabeth, Daisy, Devon, Dr. Michelle Goody, and of course Everett and Shasta, may she rest in peace. The members of this lab not only made me excited to come in each and every day, but truly taught me valuable things about science and myself. I was able to grow as a researcher and a student and I will treasure the memories of my first research lab for forever. I am forever grateful I was able to make lifelong friends I know I will always be happy to reach out to.

I would also like to extend my thanks to my committee members: Dr. Jared Talbot and Dr. Ben King, not only for their continued insight and advice surrounding my project, but also for their influence on my education. From my Bioinformatics class to the joint lab meetings and constant need for HRMA help, I always left an interaction having learned something new and I am happy to have them on my committee.

To my friends and family, this has been quite a journey for us, and I would never have gotten to this point had it not been for the love and, especially, patience I received from you all. I am forever grateful to have you all be there to catch me when I fall, to lift me up when I needed it, and especially to humble me when it was necessary. To my roommates on Middle Street

especially, I hope living with this old man wasn’t always the worst. Please just remember to clean your dishes.

Thank you to any University of Maine staff/faculty member that taught me, helped me, or was just kind to me. Thank you, Mark Nilan, for the excellence in care and maintenance of the zebrafish facility. Lastly, I would like to thank the School of Biology and Ecology for providing a wonderful education at the undergraduate and graduate levels and for the opportunity to grow as a scientist.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1:
INTRODUCTION

1.1 Skeletal Muscle Structure and Function

Skeletal muscle is one of the 3 main types of muscle found within the body, the other two being smooth and cardiac muscle. Skeletal muscle's main function is to control the motor movements the body uses for any activity such as walking, breathing, running, etc., and its structure is uniform across the entire body. However, skeletal muscle has roles in many other functions in the human body, including temperature regulation and endocrine function [1]. Overall, Skeletal muscle is an extremely important organ and proper skeletal muscle health is imperative for whole-body health and wellness.

1.1.1 Skeletal Muscle Structure

Aside from being one of the most dynamic and plastic tissues in the human body, skeletal muscle has a uniform structure that is preserved across each area of the body it occurs in [2]. Skeletal muscle can be visualized as a series of cylinders wrapped inside another series of cylinders, continuing into the molecular level (Figure 1a). For example, the bicep of the arm, the muscle that causes adduction of the forearm, is constructed of multinucleate muscle cells, called muscle fibers, bundled together in a muscle fascicle. These muscle fibers are made up of myofibrils, which are strings of the structures that produces muscle contraction: the sarcomere (Figure 1). The sarcomere is where skeletal muscle myosin interacts with F-Actin filaments to shorten the muscle; many other proteins are involved to make this contraction work, but my thesis will only focus on actin and myosin. ATP is hydrolyzed so that the myosin heads can slide along the actin filaments, shortening the sarcomere [2]. When this

process is occurs across the myofibril, a contraction is produced. Relaxation of the sarcomeres happens when Actin is no longer actively moved by myosin. This muscle relaxation allows it to extend, such as when you put the weight down and relax your arm.

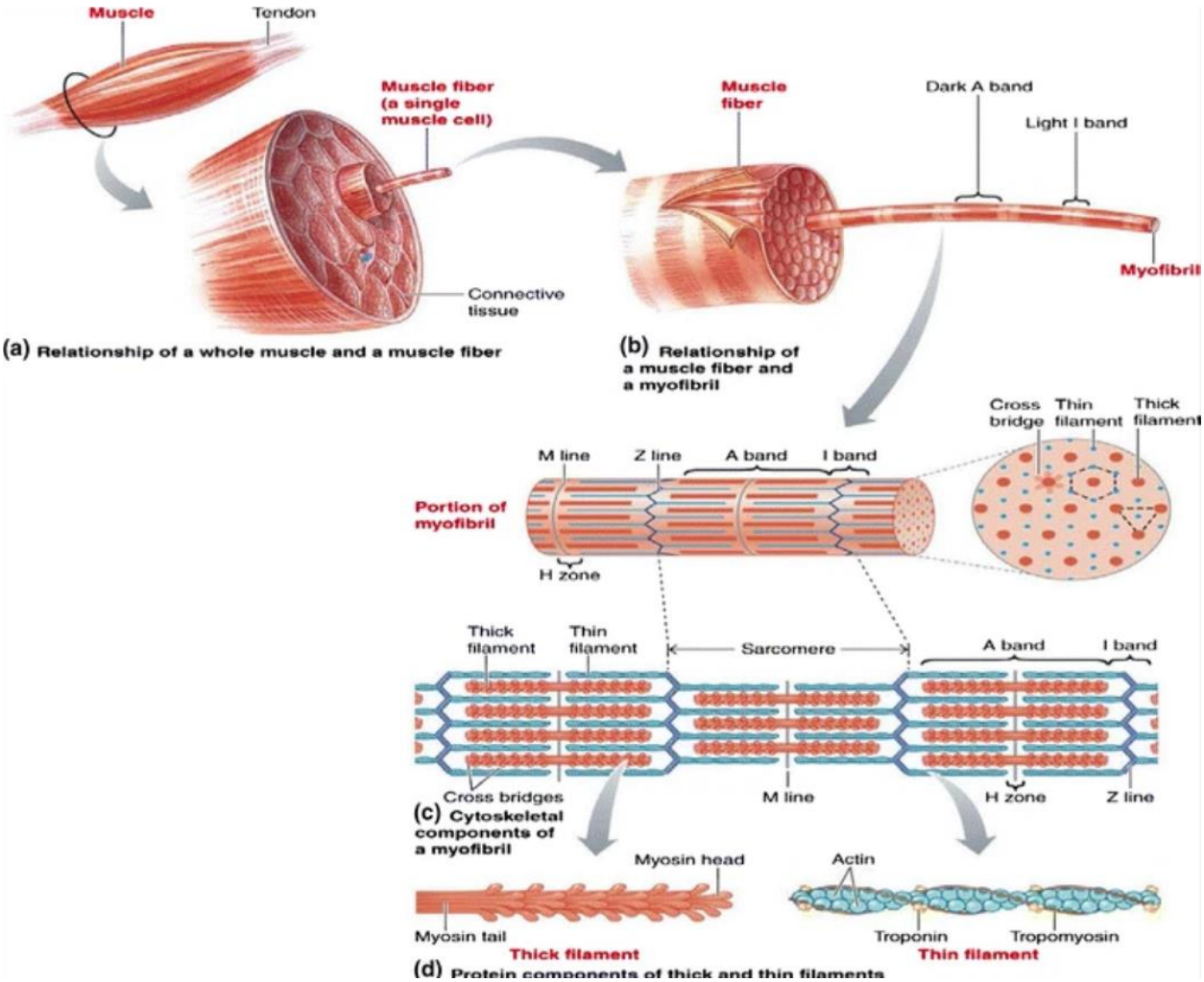

Figure 1. Skeletal muscle structure, from Frontera and Ochala, 2014. The muscle consists of a muscle fiber (a), which contains the myofibril (b). Inside each myofibril contains the basic contractile unit of skeletal muscle: the sarcomere (c). The sarcomere contains the proteins actin and myosin, which interact to perform the contraction needed to move the body (d).

1.1.2 Skeletal Muscle Function

Skeletal muscle's primary function in an organism is locomotory. Any voluntary movement requires the use of skeletal muscle. Maintaining an upward posture on two legs is

only able to be achieved via our skeletal muscle. This is done through the contraction of the muscle fibers in unison in the specific muscle being used (see section 1.1). However, movement is not the only function of the skeletal muscle; it also has metabolic functions and is vital to thermoregulation.

When an individual partakes in intense exercise, the body will start to heat up, eventually producing sweat in order to cool itself down. The heat produced from the hydrolysis of ATP travels to the bloodstream which raises overall body temperature, and triggers perspiration [1]. This regulation of the body temperature provides an advantage especially when our bodies experience a drop in temperature, also known as cold stress. Since we do not have proper insulation to deal with a cold stress, one of the solutions we employ is through our skeletal muscles. Shivering is a primary example of this. When we experience cold stress, we shiver- a process wherein many skeletal muscle groups, begin to contract rapidly at about 20% of their force-generating capacity [3]. This rapid contraction produces a large amount of heat which travels through the body and increases overall temperature, staving off the detrimental effects of cold stress.

Recent studies have also pinned skeletal muscle as a possible endocrine organ. An endocrine organ produces and secretes regulatory hormones into the blood to be transported all over the body. This is partially seen in skeletal muscle through the secretions of molecules called myokines [4]. These molecules are a type of cytokine, small secreted proteins that have a targeted effect on the interactions and communication between cells, and have autocrine, paracrine, and exocrine functions [5]. The ability to improve in structure and function while also combatting the effects of aging and disease seen in skeletal muscle requires a lot of "cross talk" between different organ systems [4]. These organs include the liver, adipose tissue,

bone, and cardiovascular system (Figure 2) [4]. Autocrine and paracrine function of myokines connect skeletal muscle to organs essential for the regulation of growth and lipid metabolism of the skeletal muscle, which provides a feedback loop used for adaptation of the muscle to exercise training [6]. Myokines also have an endocrine function that can mediate the whole-body effects of exercise further than that of the skeletal muscle, an example being the possible counteracting of the pro-inflammatory adipokines released by the adipose tissue during prolonged inactivity. The endocrine function of the skeletal muscle helps support the idea that skeletal muscle health is essential for overall health homeostasis of the body.

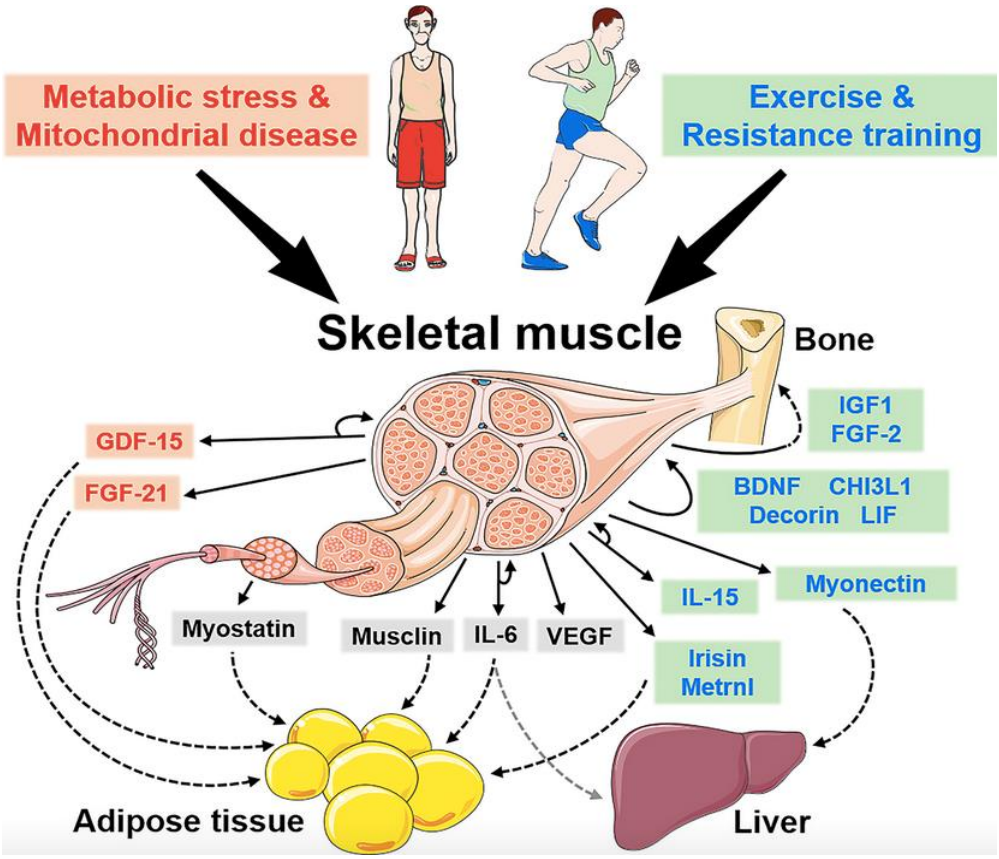

Figure 2. Stress on the skeletal muscle can induce cell signaling through the use of myokines Taken from Ost et al., 2016. The myokines produced as a result of cellular stress are highlighted in red, exercise induced myokines are highlighted in blue, and myokines produced by both conditions are highlighted in grey.

1.1.3 Attachment of the Muscle Fibers to the ECM

To effect conscious movement, the skeletal muscle contracts, generating a force on the skeletal system. These local forces can be immense, especially at times of intense exercise, potentially strong enough to tear the muscle, detaching the fiber from its neighbors or from the tendon, the band of connective tissue that connects the muscle to bone. To combat this, the muscle fibers need to be anchored properly to the extracellular matrix (ECM). They are indirectly anchored to the ECM by transmembrane receptor complexes [7]. These complexes are essential for the proper attachment of the muscle fibers to their ECM which prevents detachment during contraction. The two receptor complexes (Figure 3) in skeletal muscle fiber attachment are the integrin-mediated receptor complex and the dystrophin-glycoprotein-mediated complex. Each of these complexes consist of a variety of proteins such as dystrophin, alpha- and beta-dystroglycan, laminin, etc., and are coded for and produced by different genes. For example, the dystrophin protein (see gray boxes in figure 3) is coded by the *DMD* gene, one of the largest genes in the human genome, consisting of about 79 exons and making up roughly 0.1% of the genome [8]. Dystrophin attaches to the actin cytoskeleton, long stiff chains of actin protein that form part of the cytoskeleton and give the cell structure, with sarcoglycan proteins on the other end. The sarcoglycans consist of four transmembrane glycoproteins and respond to muscle contractions, transducing mechanical information into a cellular signal [9]. The next protein structure in the attachment complex is dystroglycan, which consists of two subunits: alpha- and beta-dystroglycan (purple structures in figure 3). Dystroglycan is then attached to laminin, one of the most widely expressed ECM proteins (yellow, stick-like structure in figure 3) [10]. It plays critical roles in embryonic development, especially the binding of the muscle attachment complex to collagen fibers. Collagen is a triple-helix molecule that

exists in many different isoforms in many places in the body [11]. In the dystrophin-glycoprotein-mediated attachment complex, all of these protein structures are connected to one another, and finally to collagen (gray proteins in Figure 3), where the fiber is secured. Without the dystrophin protein, the dystrophin-glycoprotein complex (DGC) is disrupted.

These proteins form the complexes that span from the actin cytoskeleton, through the muscle cell membrane, to the ECM. This structure can be thought of like links in a chain. A weakening of one of these links can pose a threat to the breakage of the chain, releasing whatever object is held on at the other end, which is actin in the case of muscle. When one or more of the proteins do not work properly, or have been weakened, the muscle cell can

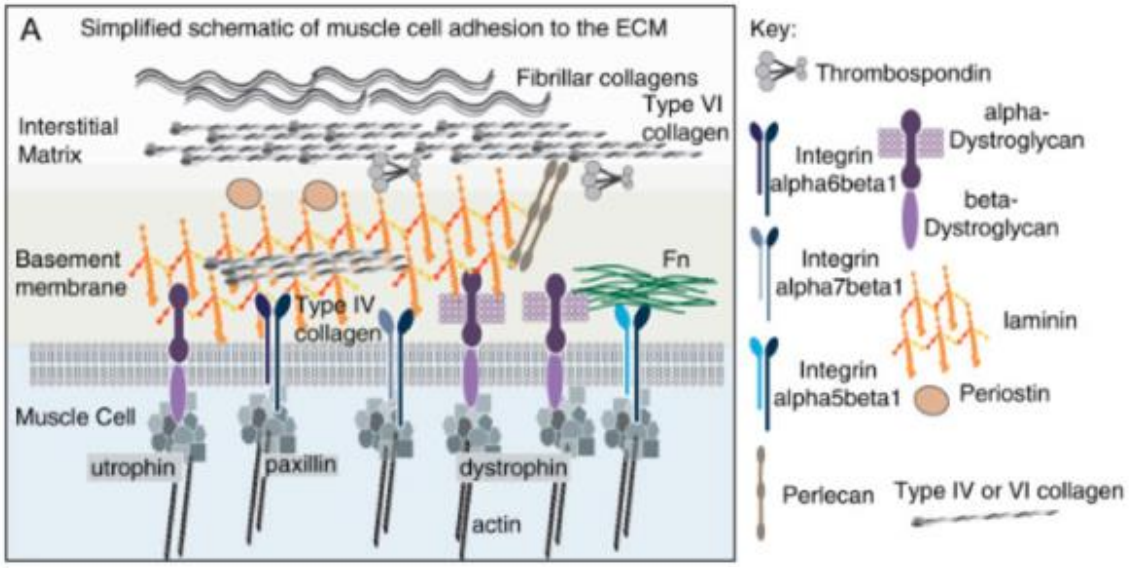

Figure 3. Cell Adhesion Complexes are Made Through Many Different Proteins, Adapted from Goody et al., 2015. Skeletal muscle cell adhesion is done through a variety of proteins. All of these proteins are coded for in different genes and a mutation in any one of those genes can cause a form of muscular dystrophy.

detach and undergo cell death. When this happens at a large scale in the muscle, we see atrophy of the muscle itself. The muscle will then attempt to rebuild broken muscle fiber cells and will replace it with a greater amount of contractile tissue, a process called hypertrophy [12].

1.1.4 Muscle Fiber Repair and Regeneration

Muscle is one of the most dynamic tissues in the body [2]. This is partially due to the normal protein turnover rate in muscle, aside from that due to damage or stress, which is about 5.7 $g \cdot kg^{-1} \cdot day^{-1}$ [13]. The rate of protein turnover increases greatly during exercise and the resulting rate depends on the type, length, and intensity of the exercise [14,15]. The increased protein turnover is essential to the repair and regeneration process that occurs in the muscle fibers after exercise. When a person takes part in intense activity, such as weightlifting, the person will probably feel a soreness in the muscle groups worked 24-48 hours after the workout. This soreness is the muscle fibers that were torn and damaged during the workout being repaired. The muscle fibers will then be repaired, and the contractile tissue will have increased, improving muscle performance that will decrease damage due to the next intense exercise. This is called muscle hypertrophy [12].

Regeneration and repair in muscle is coordinated through many different mechanisms and processes which involve cell-cell and cell-matrix interactions [16]. Muscle repair can be divided into three different phases: the demolition phase, the repair phase, and the remodeling phase [16,17]. The demolition phase is characterized by the sealing of damaged myofiber ends, inflammation, and the necrosis and clearing of damaged muscle fiber segments [17]. The clearing of necrotic muscle fiber is done through the recruitment of cells such as macrophages and neutrophils [17,18]. The next phase, the repair stage, occurs when satellite cells activate, proliferate, and differentiate to replace myofiber segments or to form new muscle fiber cells; formation of fibrous scar tissue occurs in this stage as well depending on the intensity of the damage [17]. The final stage involves muscle fiber repair in order to restore proper muscle function [17]. There typically is a positive gain in the contractile tissue

which allow for a greater force to be exerted by the muscle during the next session of exercise [19].

In people with healthy skeletal muscle, this process is repeated whenever the muscle is damaged, and proper function returns quickly. However, this process can be impaired by muscle diseases. When this happens, the overall health of the skeletal muscle tissue is decreased. This leads to a cascade of symptoms all over the body, and an eventual decrease in quality of life.

1.2 Muscle Disease

There are many diseases and disorders that negatively affect the skeletal muscle. They can stem from a variety of different origins such as neuronal, congenital, molecular, etc. One of the most common symptoms of muscle disease is atrophy: the decrease in size of the muscle fibers, eventually leading to the weakening of the muscle [20]. Depending on the severity of the disease, atrophy of the muscle can impede on everyday activities and often worsens as the person ages, eventually leading to a decrease in the length and quality of life.

1.2.1 Congenital Muscular Dystrophies

Congenital muscular dystrophies (CMDs) are a group of muscle diseases that occur when there is a change in the genetic code of the individual in a location essential to skeletal muscle health and function. These mutations in the DNA cause a loss of function in the proteins that anchor the muscle fiber to the basement membrane. When there is improper anchoring, the muscle fiber can detach and undergo cell death.

Symptoms of CMD is often initially observed in infants (although late onset is possible). Abnormal motor movements, such as trouble walking or use of their limbs, are initially seen

in young patients, followed by a progressive weakness which may lead to the loss of the ability to walk or use of limbs. However, different CMDs affect different muscle groups at a differing severity level. Some CMDs may affect the cranio-facial or ocular muscles while others effect the muscles at the limbs and shoulders. The most common CMDs are Duchenne and Becker muscular dystrophies (DMD and BMD, respectively), which effect approximately 1 in every 5,500 to 7,700 men age 5 to 24 [21]. In the case of this study, DMD will be the primary focus. However, any mutations in the DNA that code for the proteins in the adhesion complexes can cause a different CMD. Examples of these are *ITGA7* (integrin-alpha 7), *LAMA2* (laminin-alpha 2), and *COL6-RD* (collagen VI) genes.

Mutations in *ITGA7* cause a congenital myopathy due to a loss of function in the integrin proteins in the adhesion complex (figure 3). Mutations in the laminin-alpha 2 proteins coded for in the *LAMA2* gene cause *LAMA2* CMD also known as merosin-deficient congenital muscular dystrophy (MDC1A) and is characterized as early onset and very severe [22].

1.2.3 Duchenne Muscular Dystrophy

Duchenne Muscular Dystrophy (DMD) is a congenital muscular disease caused by a X-linked mutation in the *Dystrophin*. This causes a loss of function in the dystrophin protein. It is usually diagnosed at an average of about 4 years of age, with symptoms often progressing quickly [23]. Increased muscle weakness, loss of the ability to walk at a young age, respiratory and cardiac issues, and a lower quality of life are among the most common symptoms, with disease progression ultimately ending in a shortened life span.

The dystrophin gene is a very large gene, spanning about 0.1% of the genome with more than 2,200 kb [8]. Surprisingly, despite its size, the dystrophin gene only contains about 79 exons, making up only a small percentage of the gene (about 0.6%) [24]. Mutations that cause DMD

mostly stem from deletions of one or more exons [23]. This makes up about 65% of the cases, while duplications make up about 6%-10% of the case, and the rest consisting of small mutations (missense, nonsense, and splice site variations) [23]. These mutations interrupt the open reading frame (ORF) and lead to the absence of dystrophin protein throughout the body. A shift in the ORF that does not cause a complete absence of the dystrophin protein, but a partially functioning, abnormal shaped protein, you get Becker muscular dystrophy (BMD), a more mild form of DMD [25]. Without the dystrophin protein, there is detrimental effects of the muscle across a large majority of the muscle. It even extends past the skeletal muscle, as patients often show an abnormal electrocardiogram by the age of 18, indicating symptoms within the cardiac muscle and respiratory system and leading to an early death in their late teens to early twenties [26]. Disease progression in DMD and other muscle disease stress the importance of skeletal muscle health and how it is linked to overall health of the body and the need for further therapies and an eventual cure.

1.2.4 DMD Patients Have Lower Activity Levels

Movement abnormalities and difficulties are common symptoms of DMD with a loss of movement independence and the need for a wheelchair occurring by around the age of 10 [23]. Activity levels are severely reduced by this time in severe DMD patients. Without proper dystrophin function, muscle fibers are be more susceptible to damage and the regenerative abilities of myofibrils is hindered [23]. Strenuous exercise or activities that a healthy person would normally partake in such as a run or bike ride is highly advised against, as irreversible damage to the muscle fibers could ensue and exacerbate disease progression [27]. Despite the potential damage to muscle and muscle fibers, it is debated that exercise in muscle disease patients may

provide a potential therapeutic effect, since exercise in a healthy person has many benefits on overall health and wellness.

1.3 Exercise and Inactivity on Muscle Structure and Function

It is accepted widely that a normal exercise routine is imperative for the function of the human body. Numerous studies have shown that normal exercise reduces the risk for many different diseases such as coronary heart disease, hypertension, diabetes mellitus, and can extend the life expectancy of a person [28]. Alternatively, insufficient activity and sedentary lifestyles have the opposite effects. Gain of abdominal and visceral fat and a higher risk of type 2 diabetes and heart disease are among many other symptoms that may arise from the lack of regular exercise [29]. Done under proper conditions, exercise is among one of the most important things that can be done to improve health and quality of life in a healthy individual.

1.3.1 Exercise and Its Beneficial Effects on the Body

Regular exercise, especially dynamic exercise of moderate intensity, improves endothelial function, vascular tone regulation (through the increase of endothelial nitric oxide production) that staves off cardiac-related illness, and improves movement abilities [30]. In younger people, exercise shows similar benefits such as a lower heart rate, left ventricular ejection fraction, maximal oxygen uptake, and lower risk of diseases such as type 2 diabetes and heart disease [31].

When a person goes through intense exercise, they are putting strain on their muscle fibers and whether they know it or not, they are putting these fibers through a cycle of breakdown and regeneration. As explained in section 1.4, when undergoing damaging force, such as an

intense power-lifting workout, many muscle fibers break, the damaged tissue is then broken down, and contractile tissue is regenerated, often with additional tissue. The addition of new contractile tissue lets the muscle exert that same force with less damage to the muscle fibers. The consistent addition of contractile tissue due to intense exercise is called muscle hypertrophy. Athletes and power-lifters utilize hypertrophy to build muscle strength and improve performance.

1.3.2 Inactivity and Its Detrimental Effects on the Body

Regular activity clearly shows various benefits to the overall health of the body. Conversely, prolonged inactivity has been shown to have a variety of negative effects on the health and function of the body. Not meeting the minimum recommended amount of activity, which is about 30 minutes of moderate-intense physical activity 5 days a week, is a problem seen across all life stages from childhood past adulthood [29]. Prolonged inactivity not only decreases muscle mass and strength, but it is shown to be positively associated with all-cause mortality, fatal and non-fatal cardiovascular disease, type 2 diabetes, and metabolic syndrome [32].

Inactivity has a profound effect on the skeletal muscle of an individual. Increased fatiguability upon resumption of activity, loss of muscle mass, and mitochondrial dysfunction are all linked to prolonged inactivity [33–35]. When a person becomes inactive, over time there is a reduction in protein synthesis and an increase in proteolysis due to the decreased use of transcriptional activity [35]. The person is no longer using their muscle fibers beyond that of basic movement, which basically tells the body there is no use for excess proteins or transcripts of those proteins. When this happens, the body starts to breakdown those proteins

and their transcripts, ultimately diminishing the amount of muscle fibers. This is a process called muscle atrophy. With muscle atrophy, there is also a weakness of the muscles and a decreased amount of force those muscles are able to generate. About 1 week of inactivity, such as bed rest decreases as much muscle as would be gained from 12 weeks of intense resistant-type exercise training [36].

Muscle disease patients, including DMD patients, experience high levels of muscle atrophy, reducing functionality of those muscles, and lowering activity levels. Gradual resumption of activity in a healthy individual can rebuild muscle groups, reduce adipose tissue deposits, and reduce the risk for illness. However, in an individual with an illness such as muscle disease, the process of activity versus inactivity becomes more complicated.

1.3.4 Exercise and Activity and Its Effect on Muscle Disease

The main hallmark of muscle disease is increased muscle atrophy. The breakdown of muscle fibers and weakening of the muscle groups is due largely to the damage sustained to the fibers from improper anchoring to the ECM. With a lack of proper anchoring, the rate of breakdown exceeds that of the rate of regeneration, and damage to the muscle ensues. As there is no current cure for muscle disease, there is a large stress put on therapies in order to extend quality and length of life. Physical therapy is typically the most used therapy to help combat DMD and may include some type of exercise. While a current set of international care guidelines exists and recommends individuals engage in regular submaximal activities, large-scale randomized control trials are lacking, and data between smaller studies is variable [27,37–40]. For example, a study done by Voet et al. published in 2019 had participants with certain muscle diseases (DMD among them) take part in strength training, aerobic exercise,

or a combination of both in order to assess the benefits or harm to muscle strength and aerobic capacity [41]. The researchers showed uncertainty in their results and failed to show concrete evidence of improvement to muscle strength or aerobic capacity [41]. Due to limitations in human studies and variability in data, researchers have turned to animal models in order to explore and understand mechanisms behind DMD and other muscle diseases.

1.3.5 Animal Models of DMD and Exercise

Currently, there are about 60 different animal models of DMD ranging from mammalian such as canine, feline, and mouse models, to non-mammalian models including *C. elegans,* fruit fly, and zebrafish models [42]. The most widely used animal model of DMD is the *mdx* mouse, *Mus musculus*, discovered in the 1980s [42]. The mutation in the *mdx* mouse is a nonsense point mutation, C-T transition in exon 23 that aborts the full-length dystrophin expression [42,43]. Studies with exercise and *mdx* mouse have shown inconsistent results, but recent studies show more promising results. For example, a study done by Zelikovich et al. published in 2019 tested the effects of moderate and low intensity exercise on tetanic and specific force, respiratory capacity, and cardiac function [44]. They found that exercised mice *mdx* mice improved tetanic and specific force, increased respiratory capacity, and enhanced cardiac function as compared to the sedentary group. The researchers also found a dose-dependent increased in serum adiponectin, a protein hormone responsible to the influence of the body's response to insulin.

While many exercise studies of DMD have been performed, studies on inactivity and its effects on disease mechanisms and progression are lacking. It is well established that individuals suffering from DMD exhibit lower activity level compared to healthy individuals from even a very young age [45]. Two studies of inactivity in *mdx* mice show that prevention of

exercise preserves muscle fibers integrity and prevents myonecrosis [46,47]. Another study done by Li and Arner in 2015 chemically induced inactivity with BTS in *dmd* mutant zebrafish from 18 hours post fertilization (hpf) to 4 dpf which resulted in better organization of muscle fibers seen from birefringence compared to the active controls, further suggesting that inactivity helps preserve muscle fibers [48].

Conversely, a study done by Hourdé, C. *et al* in 2013 found that inactivity in *mdx* mice may lead to an increase in susceptibility to contraction-induced injury and that voluntary activity may help protect from this susceptibility [49]. Another study done on *mdx* mice by Lindsay et al. in 2021 that had a stress-induced short period of inactivity showed that inactivity was not associated with acute skeletal muscle contractile dysfunction, but high passive stiffness could be possibly attributed to a small, but significant increase in muscle fibrosis [50]. Since these findings are varied, more research is needed in order to fully understand the effects inactivity is having on DMD.

While *mdx* mice are a widely used model and allow us to research deeper into the mechanisms behind DMD without the limitations of human models, there are limitations to mice models as well. Young *mdx* mice show little weakness and live up to 80% as long as their healthy control counterparts, far longer than human DMD patients [42]. It has been hypothesized that this may be attributed to an upregulation of utrophin seen in *mdx* mice not seen in humans. Efforts to "humanize" the *mdx* model show variable results, and more research is needed in order to establish a more "human model" of mouse DMD [51]. However, other animal models are being increasingly used to study DMD, even in exercise studies. One example is zebrafish (*Danio rerio*). A study done by Kilroy et al. published in 2020 compared inactivity and neuromuscular electrical stimulation (NMES), the zebrafish version

of exercise, on a zebrafish model of DMD [45]. They found that NMES returns gene expression to wild-type levels, increases muscle adhesion to the ECM, and remodels the ECM to support more regeneration, while inactivity had a deleterious effect on disease progression. These results provide further evidence that exercise is beneficial for DMD patients and inactivity may have a negative effect on disease progression. The use of zebrafish has been increasingly used for a variety of studies and provides an attractive model to study DMD.

1.4 Zebrafish as a Model of Study

Zebrafish (*Danio Rerio*) have been widely accepted as a useful model for developmental biology and genetics experiments. Zebrafish development is rapid, as embryos fully develop their major organ systems, including cardiovascular, nervous, digestive, and muscle systems, in less than a week [52]. While they develop, the skin is transparent which makes observation and imaging of organ system development especially easy and efficient. There is also high fecundity in zebrafish; females are able to produce many offspring, potentially up to 350 offspring in a week [53]. To reproduce, zebrafish fertilization is external, which lets the embryos be collected shortly after fertilization occurs, providing researchers access to all stages of development. The high fecundity and rapid development allow for multiple experimental trials be conducted over a relatively short amount of time.

The cost of maintenance of zebrafish is relatively low as well. The embryos do not need food until 5 days post fertilization, which is the conclusion of many developmental experiments as their organs have already developed. Different transgenic or mutant lines are also able to be purchased for relatively low cost and kept for over 2 years. The small size of

the adult zebrafish also allows for multiple fish to be kept in the same tank, reducing the need for multiple tanks at large sizes, keeping maintenance costs low.

Zebrafish are also used as a model of study due to the molecular tools available and their genetic amenability. Examination of specific gene function through the development of transgenic lines, large scale mutagenesis screens, or antisense gene knockdown is highly achievable with zebrafish [52]. It has been shown by the UK Sanger institute that almost 70% of human genes have functional homologs in zebrafish, including many of the genes that code for the proteins that attach the muscle fiber to the basement membrane [54]. Genetic and functional conservation is important because that means that zebrafish display similar phenotypes resulting from the same mutations in their genome as humans. In other words, a mutation in a gene that encodes for a protein involved in muscle attachment in a zebrafish will produce similar muscle attachment deficiencies as mutations in the homologous human genes.

Two common methods of investigating gene function in the zebrafish model are morpholino-mediated protein knock down and CRISPR/cas9 mutagenesis. Morpholino (MO) antisense oligomers are synthetic oligonucleotides that interfere with mRNA translation or processing, which results in protein knock down or a “pseudo” mutant scenario. Advantages to MOs are that large numbers of “pseudo” mutants can be generated for experiments and maternal and embryonic proteins are both knocked down. Disadvantages include only temporary protein knock down, injection variability, off-target effects, and results that often don’t line up with mutant findings [55]. Alternatively, we can now relatively easily alter DNA sequences through CRISPR/cas9 mutagenesis. This system allows genome editing of different species using specific RNA segments and the Cas9 enzyme to cut the target DNA

sequence and replace it with a customized DNA sequence [54]. Research labs and institutes are using this technique to create mutant lines of zebrafish to model different genetic diseases.

1.4.1 Zebrafish Muscle Development

Zebrafish muscle development makes them an ideal model to study muscle disease. The development and physiology of skeletal muscle in zebrafish shares many commonalities with higher vertebrates, including mammals such as mouse and dog models [56]. However, there are differences in the skeletal muscle of zebrafish compared to higher vertebrates that can be viewed as experimental advantages. In zebrafish, the different muscle fiber types, fast-twitch vs slow-twitch, are spatially segregated. This allows for the observation of fiber-type specific effects when experimenting while also providing an opportunity for identifying the mechanisms that regulate fiber-type specification [56]. Development of the muscle is also easily observed. Skeletal muscle development in zebrafish is rapid and completed in a few days. The muscle originates from somitic cells as the precursors to the individual fibers. These precursor cells elongate and eventually become the actual muscle fibers, which are then separated into segments called myotomes (figure 5) [57]. These myotomes are characterized by having a sideways "V" shape, also known as a chevron, with an angle of approximately 90º. The myotendinous junction (MTJ) separates the myotomes and is the area in which attachment to the ECM occurs. Proper MTJ anchoring is essential for fiber health and overall muscle health.

1.4.2 Zebrafish Model of DMD

For this project, we used *dmd* zebrafish to model human DMD [58]. *dmd* mutant zebrafish result from a nonsense mutation that leads to a premature stop codon in the zebrafish

orthologue of the human Dystrophin gene and result in dystrophic pathology by interrupting the link between the actin cytoskeleton and the ECM in skeletal muscle cells [58,59]. The disease progression of *dmd* mutant zebrafish mimics that of a human patient very similarly as opposed to the *mdx* mouse model. Similarity to the human disease, combined with the advantages of a zebrafish model outlined in section 4 allow for a useful and potentially more relevant model of DMD. Birefringence imaging (Figure 6E) allows for easy visualization and quantification of disease progression, while tools such as DanioVision and immunohistochemistry (IHC) allow for further analysis into muscle function and structure, respectively. Overall, *dmd* mutant zebrafish provide an excellent model of DMD and are the model used to conduct this project.

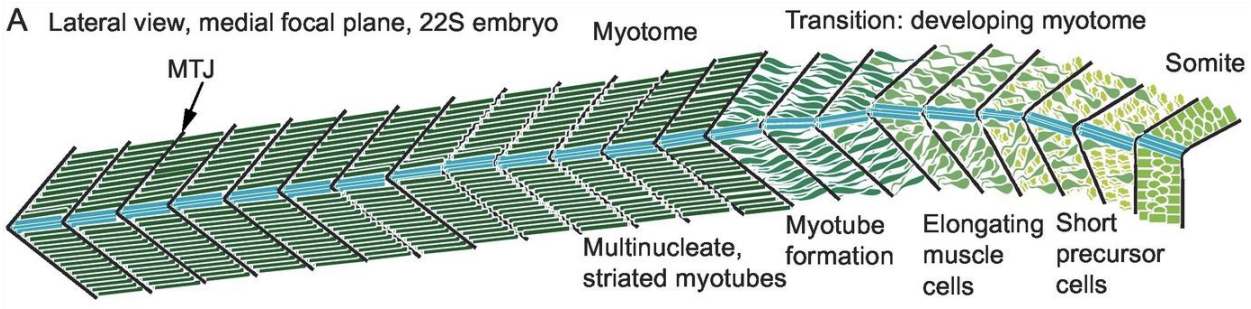

Figure 4. The Development of Skeletal Muscle from Somites: Adapted from Goody, Carter, Kilroy, Maves, & Henry, 2017. The development of skeletal muscle starts with the somite (pictured on the right) which contains short-precursor cells. These cells elongate, attach to ECM, and then form the multinucleated striated skeletal muscle fibers within the myotome (pictured to the left).

1.5 Project Overview

The sole study examining the effects of inactivity on *dmd* mutant zebrafish showed a deleterious effect on muscle structure and function [45]. This project aims to expand the research done on inactivity in DMD mutant zebrafish, to further understand the underlying mechanisms of disease progression and effects on muscle structure and function. We

hypothesize that extended inactivity will have a negative effect on the structure and function of the muscle along with a lower probability of survival in *dmd* mutant zebrafish.

Inactivity was induced through a *N*-benzyl-*p*-toluene sulphonamide (BTS) solution, an inhibitor of the Ca^{2+}-stimulated S1 ATPase that blocks gliding motility [60]. BTS has been shown to prevent any force transduction or movement in zebrafish, with little effect on the M- and Z-lines of the muscle fibers [61]. Zebrafish mutants and wild-type (WT) controls were either treated with 50 µM BTS solution, inducing inactivity, or put in a DMSO control treatment at the same concentration. Birefringence imaging was done to quantify disease severity and progression through calculation of mean grey value (MGV), DanioVision was used to analyze muscle function through measurements of distance (in mm) and velocity (in mm/s). The results of this study help to further understand the mechanisms behind inactivity and DMD in hopes to apply the data into a broader context to properly address treatments and therapies for patients diagnosed with DMD.

CHAPTER 2:

MATERIALS AND METHODS

2.1 Materials

2.1.1 1x ERM

To prepare a 20X stock solution the following was added to 800 mL of ddH2O: 17.5 g NaCl, 0.75 g KCl, 2.9 g CaCl-2H2O, 0.41 g KH2PO4, 0.142 g Na2HPO4 anhydrous, and 4.9 g MgSO4-7H2O. Once in solution, ddH2O was used to fill up to 1 L. This solution was then filter sterilized into an autoclaved 1-L flask and stored at +4 ºC. 1X ERM was prepared by adding 50 mL of the 20X stock solution, 0.3 g of NaHCO3, and one to two drops of methylene blue to 950 mL of autoclaved ddH2O. 1X ERM was stored at room temperature.

2.1.2 Tricaine

Tricaine was used to briefly halt movement in order to properly image birefringence. A stock solution of tricaine (MS-222, Sigma-Aldrich) was prepared by adding 400 mg of powered tricaine and 800 mg of Na2HP04 (Anhydrous) to 100 mL ddH20. The pH was adjusted to 7.0 if necessary, with 1M HCl or 1M NaOH. The stock solution was aliquoted and stored at -20 ºC. Working solutions were prepared daily for live imaging by adding 400 µL of the stock solution to 10 mL of 1X ERM (612 µM)

2.1.3 BTS

N-benzyl-*p*-toluene sulphonamide (BTS) was purchased from Sigma-Aldrich in a 5 mg powder. The BTS powder was dissolved in 382.64 µL DMSO to make the stock solution and was kept at -20 ºC. To achieve a 50 µM as outlined in Codina et al., 3µL of the BTS stock solution was placed in 3 mL of ERM for each well-dish [61].

2.2 Methods

2.2.1 Zebrafish Care and Maintenance

Adult zebrafish were kept on a 28.5ºC 14-h light/10-h dark cycle and embryos were collected from natural spawning of these zebrafish. We used *sapje*^{ta222a} zebrafish for all experiments [58]. Embryos were staged based on Kimmel et al., 1995 [62]. Experiments were conducted with the approval of the University of Maine Institutional Animal Care and Use Committee (IACUC). Embryo Rearing Media (ERM) was made in a 1 L bottle with 0.3 g $NaHCO_3$, 50 mL 20x ERM stock, and brought to 1 L with ddH_2O. Two drops of methylene blue were added after the ddH_2O. Embryos were housed individually at 2dpf in 4x3 12 well-plate dishes with 3 mL embryo rearing medium (ERM) during experiments. Once at 5 (dpf), zebrafish were fed once daily. At 9 dpf, ERM was switched out with system water that the adult zebrafish are housed in.

2.2.3 Birefringence Analysis

Birefringence is a unique and physical property in which light is rotated as it is passes through highly organized matter, such as the pseudo-crystalline array of muscle sarcomeres [63]. With zebrafish larvae being optically transparent, birefringence is a rudimentary and efficient way to quantify dystrophic muscle and ultimately, disease progression, as damaged muscle does not reflect light. Zebrafish were placed in 612 μM of tricaine in 1X ERM immediately prior to imaging and then transferred to a 35-mm glass bottom dish. Birefringence images were taken on a Leica MZ10 F Stereomicroscope with a Zeiss AxioCam MRm or Leica DMC5400189 camera attached. An analyzer in a rotatable mount (Leica) was attached to the objective and the glass-bottom petri dish was placed on the polarized glass stage.

Zebrafish displaying well organized myotomes with bright white or light grey muscle were classified as wild-type (WT) siblings while those that had patchy dark grey or black areas, indicating dystrophic or disorganized muscle fibers, were classified as *dmd* mutants. Imaging parameters and time was consistent across experiments.

Images were blinded before analysis and mean grey values (MGV) were calculated using FIJI software [64]. The body of the zebrafish was outlined from the 7th to the 23rd myotome using the "polygon selections" tool and then MGV was measured. This was done three times to obtain three separate measurements, and then the average was used for calculations. All birefringence data were normalized to the average WT birefringence in each imaging session. MGVs were then presented as a percentage of the average MGV. The equation is below:

$$\frac{Embryo's\ MGV}{Average\ MGV\ of\ sibling\ control} x\ 100$$

Birefringence was used to assess the change in muscle structure and quantify disease progression from 2 dpf to 8 dpf. A positive change in birefringence corresponds to an improvement in muscle structure. Conversely, a negative change in muscle structure meant there was a worsening of muscle structure.

2.2.3 DanioVision Analysis

The DanioVision system and EthoVision XT 13.0 software (Noldus Information, Inc) was used to conduct high-throughput locomotion tracking studies to observe and quantify muscle function during inactivity at 5 dpf and 8 dpf. DanioVision uses a high-speed, infrared-sensitive camera to track movement of zebrafish. During the experiments, zebrafish were kept in their 12-well plate wells and placed into the DanioVision observation chamber. The temperature was kept

at 28 ºC and a temperature control unit was used to ensure the temperature stayed constant. Zebrafish were allowed to acclimate to the temperature for 5 minutes. Using the EthoVision software, the zebrafish went into a period of dark followed by two light-on/off cycles, where the white light was turned on at 100% intensity for 5 minutes and then turned off for 5 minutes [45]. Total recording time was 25 minutes.

Once recording was finished, the data was exported into an Excel file. Raw data includes distance swam in mm and mean velocity across 0.033-second periods. Distance and velocity were analyzed in GraphPad Prism 9.

2.2.4 BTS Inactivity Experiment

Zebrafish embryos were collected and grown until 2 dpf, the day of disease onset. At 2 dpf, birefringence images were taken as described in section 2.2.3. The embryos were then put into individual well dishes in a 12-well plate, separated by treatment group. There were 4 treatment groups: WT Inactive, WT, *dmd* Mutant Inactive, and *dmd* mutant. The BTS-induce inactive groups received 3 µL BTS stock solution in 3 mL ERM. The CT groups were treated with 3 µL DMSO. From 2 dpf to 5 dpf, the ERM solutions were changed daily at approximately the same time. At 5 dpf, the zebrafish were removed from the treatment groups and birefringence images were taken. The zebrafish then were placed into 3 mL ERM with no treatment solution. DanioVision was done 4 hours later to allow proper time for activity to resume. ERM was changed daily at approximately the same time until 8 dpf. At 8 dpf, birefringence images were taken and DanioVision was done 4 hours later. The embryos were then tracked for survival and deaths were recorded as days post fertilization.

2.2.5 Statistical Analysis

All statistical analysis was done in GraphPad Prism 9. Normality of the data was first tested using the Shapiro-Wilk test. If the data set passed the normality test, an unpaired two-tailed t test was performed between two data sets (i.e., *dmd* inactive mutants versus *dmd* mutant controls). An ordinary one-way ANOVA followed by a Turkey's multiple comparison test was performed between the four treatment groups' data sets. If the data set failed to pass the normality test, a Mann-Whitney U test was performed to compare two data sets while a Kruskal-Wallis test was performed to compare the four treatment groups' data sets. Significance for all tests was set to $p < 0.05$.

CHAPTER 3:

RESULTS

3.1 Transient BTS Treatment Does Not Alter Muscle Structure in *dmd* Mutant Zebrafish

We sought to analyze the change in muscle structure in *dmd* mutants as compared to the control treatment groups through calculation of percent MGV in order to quantify the dystrophic muscle. We found that at 2 dpf, 5 dpf, and 8 dpf, there was no significant difference between the *dmd* mutant inactive and *dmd* mutant groups (Figure 6A, B, C,). Also, there was no significant different between the WT and WT inactive groups at 2 dpf and 8 dpf (Figure 6A, and C).

Over the three time periods, we saw a decrease in MGV from 2 dpf to 5 dpf in the WT Inactive, *dmd* mutant inactive, and *dmd* mutant groups, followed by an increase in MGV in the WT Inactive group only (Figure D). The *dmd* mutant and *dmd* mutant inactive groups followed a similar trend throughout all time groups (Figure D). WT MGV did not change over the time periods (Figure 6 D). These findings provide evidence that the *dmd* mutant inactive zebrafish

followed the same disease progression as the active *dmd* mutant zebrafish over time, suggesting that inactivity is neither hindering nor improving muscle structure in this mutant. Also, these data shows us that inactivity has a detrimental effect on WT muscle that can be recovered shortly upon resumption of activity.

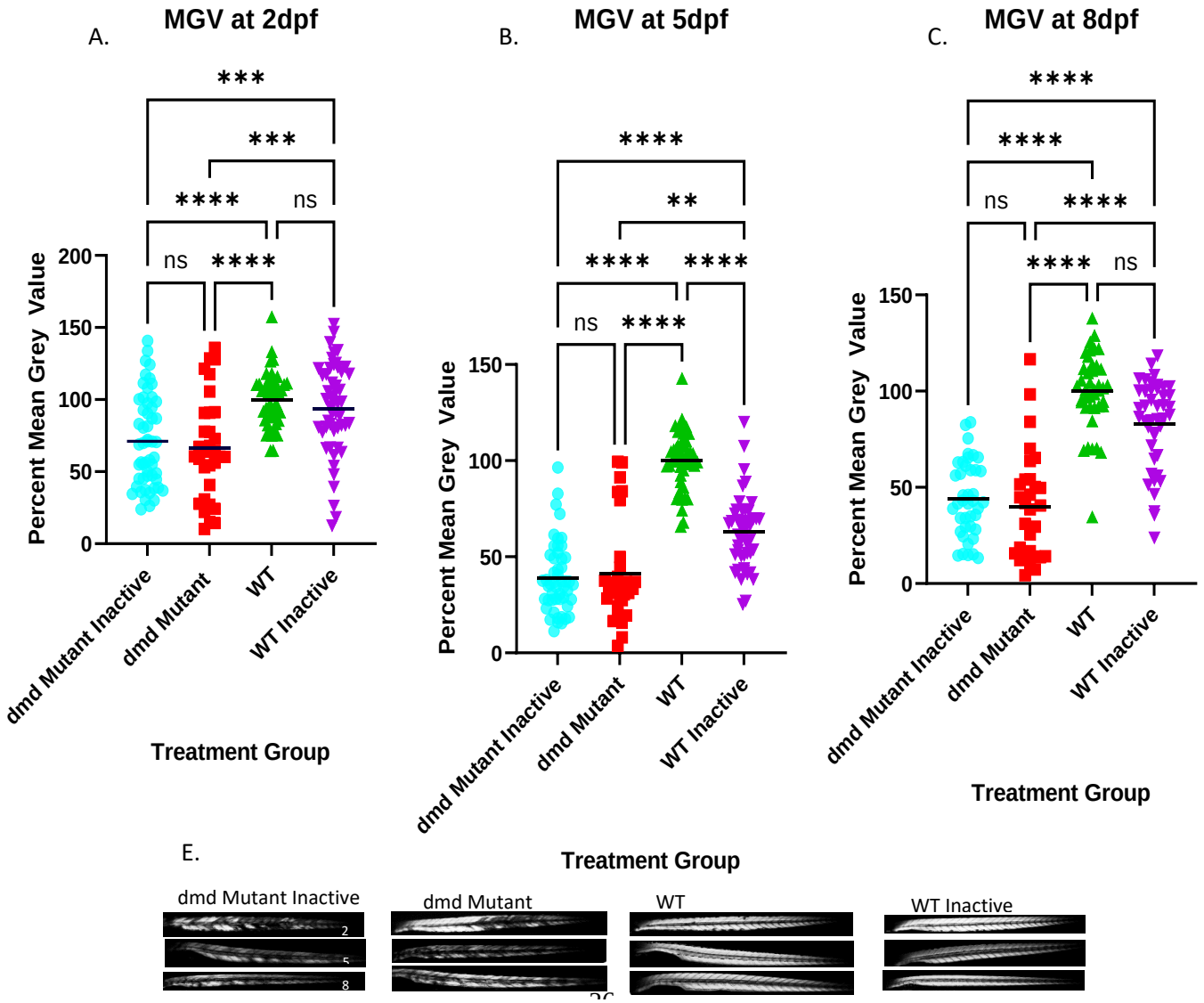

Figure 5. Inactivity does not have a negative effect on muscle structure. (A.) Percent MGV at 2 dpf shows no significant different difference in percent MGV between the dmd Mutant and dmd Mutant Inactive groups as well as the WT and WT Inactive groups. (B., C.) Percent MGV at 5 dpf as well as 8 dpf shows no significant difference between the dmd Mutant and

3.2 Muscle Function in *dmd* Mutant Zebrafish is not Affected By BTS-Induced Inactivity.

We next sought to understand the effects of inactivity on muscle structure in the *dmd* inactive mutants as compared to the CT treatment groups. We used DanioVision to measure distance (in mm) and velocity (in mm/s) in order to quantify swimming ability and attribute that to muscle function. We saw no significant difference in distance or velocity between the *dmd* mutant inactive and *dmd* mutant groups as well as the WT inactive and dmd mutant groups at 5 dpf (Figure 7A, B). At 8 dpf, the only significant difference in distance was between the WT inactive and the *dmd* mutant inactive groups (Figure 7C), while with velocity, the only significant difference was between the *dmd* mutant groups and the WT groups (Figure 7D). These findings show that muscle function analyzed through distance and velocity was not different between the *dmd* mutant and *dmd* inactive mutants at either 5 dpf or 8 dpf, suggesting that inactivity does not have a detrimental effect nor an improvement in muscle function.

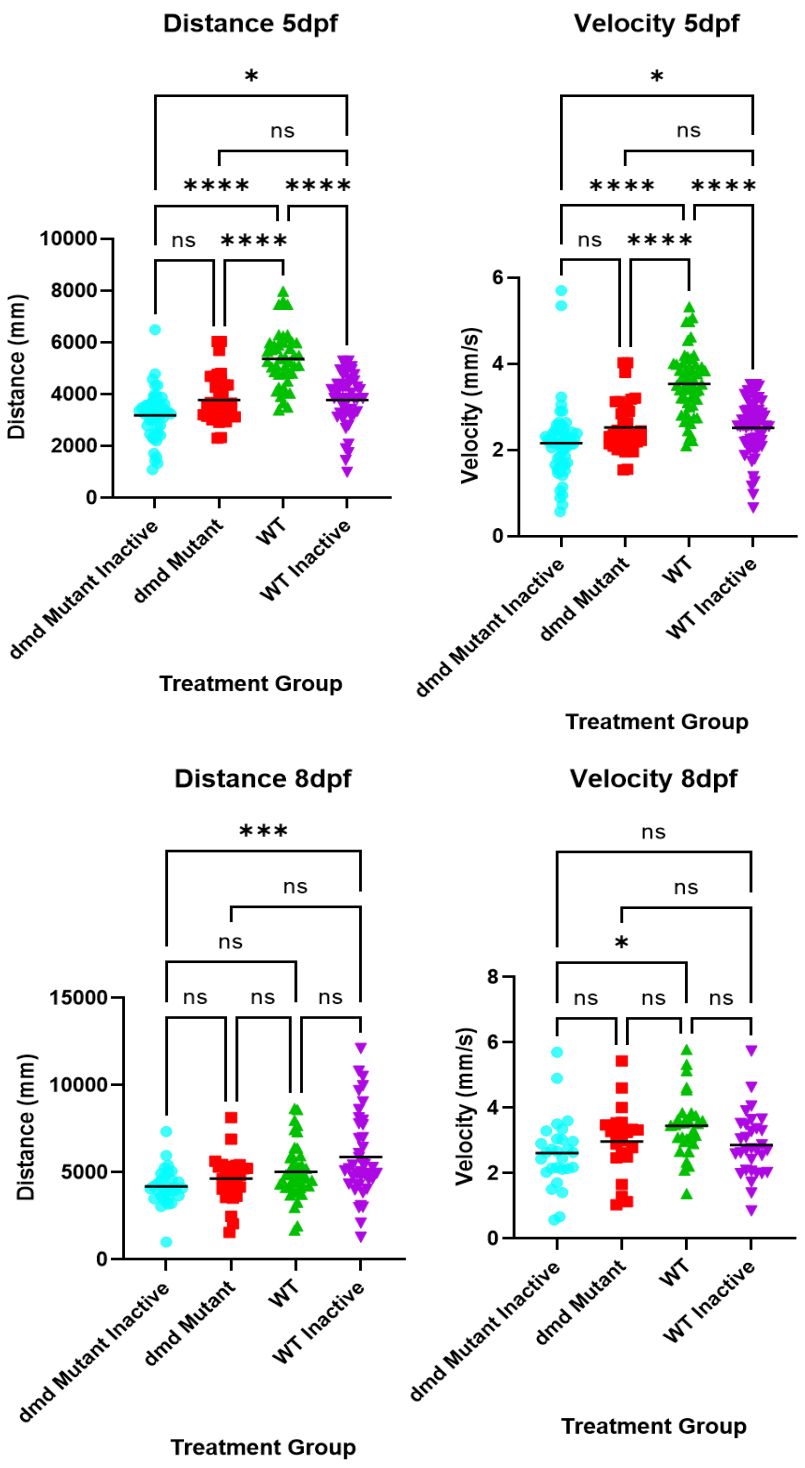

Figure 6. Inactivity does not have a negative effect on muscle function via distance and velocity. DMSO sapjes were not significantly different in distance or velocity from BTS sapjes at 5 dpf (A and B). At 8 dpf, the only groups that were significantly different was the BTS sapjes and the WT BTS group for distance (C) and the BTS sapje and the WT DMSO for velocity (D).

CHAPTER 4:

DISCUSSION

After analysis of the percent MGV throughout the time periods, we did not see a significant difference between the *dmd* mutant group and the *dmd* mutant inactive group (Figure 6A-C). This shows that while inactivity is not making *dmd* inactive mutants worse than the control mutants, it is not helping the *dmd* inactive mutants improve in muscle structure. Often times, a patient suffering from DMD becomes inactive or is told to reduce activity to simply going about their daily life, in order to preserve the muscle fibers. Our data did not provide any evidence that inactivity preserves muscle fibers and disease progression follows a similar pattern to the control mutant group. Conversely, previous data by Kilroy et al. show a worsening effect on muscle structure after the same period of inactivity when compared to the control mutant group [45]. The difference in our data could be attributed to the chemical used to induce inactivity. Kilroy et al. used tricaine (MS-222) for their inactivity studies. Tricaine works by blocking the voltage-sensitive sodium channels, preventing a voltage-dependent increase in sodium conductance, and ultimately blocking muscle contraction [65]. Adverse effects are seen mostly in the CNS and cardiovascular system; however, it is not yet fully understood. A common response to zebrafish treatment with tricaine is elevation of heart rate and respiration, followed by a depression of cardiovascular and respiratory function, resulting in hypoxia, a lowered concentration of blood O_2 [65]. While not a direct effect on the musculature, lowered blood concentration and changes in heart rate could have residual effects on already damaged muscle due to *dmd* that are not seen in BTS treatment in zebrafish.

Another study similar to what was done in this thesis is by Li and Arner et al. in 2015. The researchers here used 50 µM of BTS to induce inactivity in *dmd* mutant zebrafish at 18 hpf

and was done through 4 dpf. They found that the BTS treated *dmd* mutants displayed higher birefringence when compared to the DMSO control *dmd* mutants and was confirmed through quantification of birefringence intensity [48]. These data contradict what was found in this thesis, as we did not see an improvement in the muscle structure when compared to *dmd* mutant controls. One of the reasons for this could possibly be attributed to time of treatment. Whereas our *dmd* mutants entered BTS-induced inactivity at 2 dpf, the BTS-induced inactivity treatment in Li and Arner's experiments began at 18 hpf. At this time, zebrafish are not free swimming, as muscle development and organization is still occurring [57]. At 2 dpf, the muscle has been developed and the larvae are free swimming, opening up an increased opportunity for contractile-induced damage, especially in *dmd* mutant zebrafish, that would otherwise not occur with inactivity beginning at 18 hpf. This would preserve muscle fiber integrity starting at 18 hpf and since the researchers did not look at muscle birefringence after resumption of activity past one day post BTS washout, it is likely that there would be no evidence of dystrophic muscle within the time of treatment. It is important to wait until disease onset to initiate the treatment groups because DMD patients often first get diagnosed after there is already damage due to normal activity levels (typically in children). The *dmd* mutant zebrafish in the research presented in this thesis started out with dystrophic muscle due to their swimming levels being higher prior to BTS treatment and data was also collected 3 days post-BTS washout, which could account for the difference in results.

We also saw that WT inactive muscle structure was significantly decreased from 2 dpf to 5 dpf. This negative effect of inactivity was what we expected to happen, however, it was not a lingering effect, as structure improved to WT levels after 3 days of activity resumption. This shows us that the experimental conditions were working, and inactivity negatively effects muscle

structure. Unfortunately, we did not see this negative effect with the *dmd* mutant inactive group when compared to the *dmd* mutant. This could possibly be attributed to the fact that the *dmd* mutant inactive embryos were already suffering from their muscle weakness initiated by *dmd*. Since the WT inactive embryos were at similar levels to *dmd* mutants after their treatment, *dmd* mutants possibly already were suffering from the impacts of *dmd* that manifested worse than the effects of inactivity.

In BTS treatments, there have been reports of disruption of the thick and thin filament organization in zebrafish, as BTS works through inhibiting myosin function through the blockage of ATPase activity and interaction with α-actin [61]. This disruption in the interaction with α-actin causes disruption with the organization of the thick and thin filaments, with little effect on the M- and Z-lines [61]. This disorganization may account for lower percent MGV measurements in BTS treatment groups, as birefringence imaging relies on highly organized structures, such as the sarcomere, to reflect light and glow under the polarized lenses. While this is something to consider, we saw that the *dmd* mutant inactive group had similar percent MGV measurements as the *dmd* mutant group and were not significantly different at any of the three time periods. Therefore, it is unlikely there was a significant effect on the disorganization of the thick and thin filaments that could be seen with birefringence. While it would be difficult to observe the organization of the thick and thin filaments at the current time, immunohistochemistry staining with phalloidin would fluorescently label the actin and would be a first step in seeing how BTS may be affecting the thick and thin filament organization.

There was also no significant difference in the muscle function, via swimming distance and velocity, between the *dmd* inactive mutants and the *dmd* mutants at 5dpf. This shows that immediately after a period of inactivity, swimming ability was no different than the control

mutants who were not inactive. The lack of a significant difference could be attributed to the differences in mild versus severe phenotype of the disease. DMD can have a varying severity and the embryos were not separated based on mild versus severe phenotypes. It is possible that one group may have had more severe mutants than another, and vice versa. This may have shown an evening-out effect of the distance and velocity between the BTS mutant group and the DMSO mutant group. Percent MGV calculations can be used to separate groups and observe differences in mild versus severe phenotypes. This can be done to see if the difference in phenotype could be affecting the swimming distance and velocity.

Also, the lack of significant difference between the WT inactive group and the *dmd* mutant group at 5 dpf in both distance and velocity could be attributed to the decrease in muscle mass resulting from inactivity. In a healthy individual, a period of inactivity reduces muscle mass and strength. Since the muscle is no longer being used, the proteins are broken down and the amount of contractile tissue is reduced. This can be seen in the WT inactive group. Since they are inactive, the muscle function is reduced to the levels similar to diseased, dystrophic muscle. Resumption of activity increases muscle function back to WT levels. This data helps to support our model of inactivity.

At 8 dpf, there is no significant difference between any group other than the BTS sapje and WT inactive groups with distance and WT and *dmd* mutant groups with velocity (Figure 7C). This could be due to improvement in swimming abilities in the inactive groups. There may be an increase in distance and velocity from 5 to 8 dpf in the inactive groups that could account for the lack of significant difference between the groups, since there was 3 days allowed to recover and train the body to move about. However, it is difficult to accurately compare the changes in distance and velocity from 5 dpf to 8 dpf due to one replicate at 8 dpf not being

conducted because of having to quickly quarantine due to a COVID-19 scare along with another replicate's data at 8 dpf not being saved properly on the DanioVision computer. All other experiments were conducted without problems. Further study into the change in distance and velocity may help shed light on the lack of significant differences between the groups. Regardless, there is still no significant difference between *the dmd* mutant group and the *dmd* mutant inactive group, showing that inactivity was not making muscle function worse than the mutant controls.

This study can be improved in multiple ways. One of the ways that would improve the study the most would be separation of mild versus severe phenotypes. Mild versus severe can be calculated from percent MGV and embryos can be divided more evenly amongst the groups. This would potentially reduce the variation in muscle function and structure, along with survival that could possibly have skewed our results and provide a clearer picture of what may be happening as a result of inactivity. Also, human DMD shows variability in the severity as well, so separation of the mild and severe zebrafish would allow for a better parallel to the human disease.

In combination with separation of mild versus severe, another way of improving the study would be to collect MGV data over each day, as opposed to the three time points. This would allow us to see the change in muscle structure over time more clearly. Kilroy et al. 2020 saw a difference in the change over time in percent MGV between mild and severe at 5 dpf to 8 dpf [45]. She saw that the mild inactive *dmd* mutants, while having higher percent MGV, experienced a more dramatic decline in their percent MGV after entering the recovery period at 5 dpf. The severe inactive *dmd* mutants, having lower percent MGV, had a less dramatic decrease, but ended up reaching the same MGV levels at 8 dpf as the mild inactive *dmd* mutants. It would

definitely improve this study to see if there was the same pattern in the inactive *dmd* mutants in this project as well. Also, using DanioVision of the DMSO groups each day starting at 2 dpf and DanioVision of the BTS groups each day starting at 5 dpf (since there would be no movement in the BTS treatment from 2 dpf to 5 dpf) to analyze changes in muscle function over time could show how muscle function changes day by day. Collecting data over more timepoints overall would provide more information on how the disease progression is changing over time.

Immunohistochemistry staining of the muscle actin as well as the neuromuscular junctions (NMJ) would allow for a more in-depth look at the muscle physiology. Since Kilroy et al. induced inactivity through the neurons, it would be helpful to observe any differences in the NMJs between tricaine-treated and BTS-treated zebrafish. This could potentially explain differences in the data from the two studies. Furthermore, staining actin and myosin molecules would allow us to see if there was any disorganization of the thick and thin filaments as was previously shown to be attributed to BTS treatments. This would negate any potential decrease in percent MGV due to disorganization of these filaments and not the muscle disease phenotype itself.

From the data we collected, we can conclude that BTS-induced inactivity does not have a negative effect on the disease progression in DMD mutant zebrafish. Muscle function and structure, along with survival, was not significantly different in the BTS mutant zebrafish as compared to the DMSO mutant zebrafish. The inactive mutants seemed to follow a similar disease progression as the control mutants. In a broader context, this provides evidence that inactivity to preserve muscle fiber integrity in DMD patients may not be helping stave off the effects of DMD. It is imperative that more research be done with inactivity and muscle disease

so that proper treatments and therapies can be applied to those diagnosed with any form of muscle disease to extend and improve the length and quality of life.

BIBLIOGRAPHY

1. González-Alonso, J., Quistorff, B., Krustrup, P., Bangsbo, J. & Saltin, B. Heat production in human skeletal muscle at the onset of intense dynamic exercise. *J. Physiol.* **524**, 603–615 (2000).

2. Frontera, W. R. & Ochala, J. Skeletal muscle: a brief review of structure and function. *Calcif. Tissue Int.* **96**, 183–195 (2015).

3. Research, I. of M. (US) C. on M. N., Marriott, B. M. & Carlson, S. J. *Muscle Metabolism and Shivering During Cold Stress*. *Nutritional Needs In Cold And In High-Altitude Environments: Applications for Military Personnel in Field Operations* (National Academies Press (US), 1996).

4. Ost, M., Coleman, V., Kasch, J. & Klaus, S. Regulation of myokine expression: Role of exercise and cellular stress. *Free Radic. Biol. Med.* **98**, 78–89 (2016).

5. Zhang, J.-M. & An, J. Cytokines, Inflammation and Pain. *Int. Anesthesiol. Clin.* **45**, 27–37 (2007).

6. Huh, J. Y. The role of exercise-induced myokines in regulating metabolism. *Arch. Pharm. Res.* **41**, 14–29 (2018).

7. Goody, M. F., Sher, R. B. & Henry, C. A. Hanging on for the ride: adhesion to the extracellular matrix mediates cellular responses in skeletal muscle morphogenesis and disease. *Dev. Biol.* **401**, 75–91 (2015).

8. Gao, Q. & McNally, E. M. The Dystrophin Complex: structure, function and implications for therapy. *Compr. Physiol.* **5**, 1223–1239 (2015).

9. Tarakci, H. & Berger, J. The sarcoglycan complex in skeletal muscle. *Front. Biosci. Landmark Ed.* **21**, 744–756 (2016).

10. Yao, Y. Laminin: loss-of-function studies. *Cell. Mol. Life Sci. CMLS* **74**, 1095–1115 (2017).

11. Bella, J. Collagen structure: new tricks from a very old dog. *Biochem. J.* **473**, 1001–1025 (2016).

12. Paul, A. C. & Rosenthal, N. Different modes of hypertrophy in skeletal muscle fibers. *J. Cell Biol.* **156**, 751–760 (2002).

13. Poortmans, J. R., Carpentier, A., Pereira-Lancha, L. O. & Lancha, A. Protein turnover, amino acid requirements and recommendations for athletes and active populations. *Braz. J. Med. Biol. Res.* **45**, 875–890 (2012).

14. Rose, A. J. & Richter, E. A. Regulatory mechanisms of skeletal muscle protein turnover during exercise. *J. Appl. Physiol.* **106**, 1702–1711 (2009).

15. Pikosky, M. A. *et al.* Aerobic Exercise Training Increases Skeletal Muscle Protein Turnover in Healthy Adults at Rest. *J. Nutr.* **136**, 379–383 (2006).

16. Laumonier, T. & Menetrey, J. Muscle injuries and strategies for improving their repair. *J. Exp. Orthop.* **3**, (2016).

17. Milner, D. J. & Cameron, J. A. Muscle Repair and Regeneration: Stem Cells, Scaffolds, and the Contributions of Skeletal Muscle to Amphibian Limb Regeneration. in *New Perspectives in Regeneration* (eds. Heber-Katz, E. & Stocum, D. L.) 133–159 (Springer, 2013). doi:10.1007/82_2012_292.

18. Novak, M. L., Weinheimer-Haus, E. M. & Koh, T. J. Macrophage Activation and Skeletal Muscle Healing Following Traumatic Injury. *J. Pathol.* **232**, 344–355 (2014).

19. Schoenfeld, B. J. The Mechanisms of Muscle Hypertrophy and Their Application to Resistance Training. *J. Strength Cond. Res.* **24**, 2857–2872 (2010).

20. Bonaldo, P. & Sandri, M. Cellular and molecular mechanisms of muscle atrophy. *Dis. Model. Mech.* **6**, 25–39 (2013).

21. Romitti, P. A. *et al.* Prevalence of Duchenne and Becker Muscular Dystrophies in the United States. *Pediatrics* **135**, 513–521 (2015).

22. Yurchenco, P. D., McKee, K. K., Reinhard, J. R. & Rüegg, M. A. Laminin-deficient muscular dystrophy: Molecular pathogenesis and structural repair strategies. *Matrix Biol.* **71–72**, 174–187 (2018).

23. Falzarano, M. S., Scotton, C., Passarelli, C. & Ferlini, A. Duchenne Muscular Dystrophy: From Diagnosis to Therapy. *Molecules* **20**, 18168–18184 (2015).

24. Blake, D. J., Weir, A., Newey, S. E. & Davies, K. E. Function and Genetics of Dystrophin and Dystrophin-Related Proteins in Muscle. *Physiol. Rev.* **82**, 291–329 (2002).

25. Wilson, K. *et al.* Duchenne and Becker muscular dystrophies: a review of animal models, clinical endpoints, and biomarker quantification. *Toxicol. Pathol.* **45**, 961–976 (2017).

26. Nowak, K. J. & Davies, K. E. Duchenne muscular dystrophy and dystrophin: pathogenesis and opportunities for treatment. *EMBO Rep.* **5**, 872–876 (2004).

27. Spaulding, H. R. & Selsby, J. T. Is Exercise the Right Medicine for Dystrophic Muscle? *Med. Sci. Sports Exerc.* **50**, 1723–1732 (2018).

28. Singh, R. The Importance of Exercise as a Therapeutic Agent. *Malays. J. Med. Sci. MJMS* **9**, 7–16 (2002).

29. González, K., Fuentes, J. & Márquez, J. L. Physical Inactivity, Sedentary Behavior and Chronic Diseases. *Korean J. Fam. Med.* **38**, 111–115 (2017).

30. Garatachea, N. *et al.* Exercise Attenuates the Major Hallmarks of Aging. *Rejuvenation Res.* **18**, 57–89 (2015).

31. Sijie, T., Hainai, Y., Fengying, Y. & Jianxiong, W. High intensity interval exercise training in overweight young women. *J. Sports Med. Phys. Fitness* **52**, 255–262 (2012).

32. Rollo, S., Gaston, A. & Prapavessis, H. Cognitive and Motivational Factors Associated with Sedentary Behavior: A Systematic Review. *AIMS Public Health* **3**, 956–984 (2016).

33. Kirwan, R. *et al.* Sarcopenia during COVID-19 lockdown restrictions: long-term health effects of short-term muscle loss. *GeroScience* **42**, 1547–1578 (2020).

34. Bogdanis, G. C. Effects of Physical Activity and Inactivity on Muscle Fatigue. *Front. Physiol.* **3**, (2012).

35. Hyatt, H., Deminice, R., Yoshihara, T. & Powers, S. K. Mitochondrial dysfunction induces muscle atrophy during prolonged inactivity: a review of the causes and effects. *Arch. Biochem. Biophys.* **662**, 49–60 (2019).

36. Dirks, M. L. *et al.* One Week of Bed Rest Leads to Substantial Muscle Atrophy and Induces Whole-Body Insulin Resistance in the Absence of Skeletal Muscle Lipid Accumulation. *Diabetes* **65**, 2862–2875 (2016).

37. SICILIANO, G., SCHIRINZI, E., SIMONCINI, C. & RICCI, G. Exercise therapy in muscle diseases: open issues and future perspectives. *Acta Myol.* **38**, 233–238 (2019).

38. Bushby, K. *et al.* Diagnosis and management of Duchenne muscular dystrophy, part 1: diagnosis, and pharmacological and psychosocial management. *Lancet Neurol.* **9**, 77–93 (2010).

39. Moreira-Marconi, E. *et al.* WHOLE-BODY VIBRATION EXERCISE IS WELL TOLERATED IN PATIENTS WITH DUCHENNE MUSCULAR DYSTROPHY: A SYSTEMATIC REVIEW. *Afr. J. Tradit. Complement. Altern. Med. AJTCAM* **14**, 2–10 (2017).

40. Hind, D. *et al.* Aquatic therapy for children with Duchenne muscular dystrophy: a pilot feasibility randomised controlled trial and mixed-methods process evaluation. *Health Technol. Assess. Winch. Engl.* **21**, 1–120 (2017).

41. Voet, N. B., van der Kooi, E. L., van Engelen, B. G. & Geurts, A. C. Strength training and aerobic exercise training for muscle disease. *Cochrane Database Syst. Rev.* **2019**, (2019).

42. McGreevy, J. W., Hakim, C. H., McIntosh, M. A. & Duan, D. Animal models of Duchenne muscular dystrophy: from basic mechanisms to gene therapy. *Dis. Model. Mech.* **8**, 195–213 (2015).

43. Sicinski, P. *et al.* The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. *Science* **244**, 1578–1580 (1989).

44. Zelikovich, A. S., Quattrocelli, M., Salamone, I. M., Kuntz, N. L. & McNally, E. M. Moderate exercise improves function and increases adiponectin in the mdx mouse model of muscular dystrophy. *Sci. Rep.* **9**, 5770 (2019).

45. Kilroy, E. A. *et al.* Deleterious impacts of inactivity and beneficial impacts of neuromuscular electrical stimulation on muscle structure and function in the zebrafish model of Duchenne Muscular Dystrophy. *bioRxiv* 2020.09.02.279513 (2020) doi:10.1101/2020.09.02.279513.

46. Mizuno, Y. Prevention of myonecrosis in mdx mice: effect of immobilization by the local tetanus method. *Brain Dev.* **14**, 319–322 (1992).

47. Mokhtarian, A., Lefaucheur, J. P., Even, P. C. & Sebille, A. Hindlimb immobilization applied to 21-day-old mdx mice prevents the occurrence of muscle degeneration. *J. Appl. Physiol. Bethesda Md 1985* **86**, 924–931 (1999).

48. Li, M. & Arner, A. Immobilization of Dystrophin and Laminin α2-Chain Deficient Zebrafish Larvae In Vivo Prevents the Development of Muscular Dystrophy. *PLoS ONE* **10**, (2015).

49. Hourdé, C. *et al.* Voluntary physical activity protects from susceptibility to skeletal muscle contraction-induced injury but worsens heart function in mdx mice. *Am. J. Pathol.* **182**, 1509–1518 (2013).

50. Lindsay, A. *et al.* Some dystrophy phenotypes of dystrophin-deficient mdx mice are exacerbated by mild, repetitive daily stress. *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* **35**, e21489 (2021).

51. Yucel, N., Chang, A. C., Day, J. W., Rosenthal, N. & Blau, H. M. Humanizing the mdx mouse model of DMD: the long and the short of it. *Npj Regen. Med.* **3**, 1–11 (2018).

52. Sarasamma, S. *et al.* Zebrafish: A Premier Vertebrate Model for Biomedical Research in Indian Scenario. *Zebrafish* **14**, 589–605 (2017).

53. Steffen, L. S. *et al.* Zebrafish orthologs of human muscular dystrophy genes. *Bmc Genomics* **8**, 79 (2007).

54. Liu, J. *et al.* CRISPR/Cas9 in zebrafish: an efficient combination for human genetic diseases modeling. *Hum. Genet.* **136**, 1–12 (2017).

55. Blum, M., De Robertis, E. M., Wallingford, J. B. & Niehrs, C. Morpholinos: Antisense and Sensibility. *Dev. Cell* **35**, 145–149 (2015).

56. Goody, M. F., Carter, E. V., Kilroy, E. A., Maves, L. & Henry, C. A. 'Muscling' Throughout Life: Integrating Studies of Muscle Development, Homeostasis, and Disease in Zebrafish. *Curr. Top. Dev. Biol.* **124**, 197–234 (2017).

57. Snow, C. J., Peterson, M. T., Khalil, A. & Henry, C. A. Muscle development is disrupted in zebrafish embryos deficient for Fibronectin. *Dev. Dyn. Off. Publ. Am. Assoc. Anat.* **237**, 2542–2553 (2008).

58. Bassett, D. & Currie, P. D. Identification of a Zebrafish Model of Muscular Dystrophy. *Clin. Exp. Pharmacol. Physiol.* **31**, 537–540 (2004).

59. Li, M., Andersson-Lendahl, M., Sejersen, T. & Arner, A. Muscle dysfunction and structural defects of dystrophin-null sapje mutant zebrafish larvae are rescued by ataluren treatment. *FASEB J.* **28**, 1593–1599 (2014).

60. Cheung, A. *et al.* A small-molecule inhibitor of skeletal muscle myosin II. *Nat. Cell Biol.* **4**, 83–88 (2002).

61. Codina, M., Li, J., Gutiérrez, J., Kao, J. P. Y. & Du, S. J. Loss of Smyhc1 or Hsp90α1 Function Results in Different Effects on Myofibril Organization in Skeletal Muscles of Zebrafish Embryos. *PLoS ONE* **5**, (2010).

62. Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. & Schilling, T. F. Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253–310 (1995).

63. Smith, L. L., Beggs, A. H. & Gupta, V. A. Analysis of skeletal muscle defects in larval zebrafish by birefringence and touch-evoke escape response assays. *J. Vis. Exp. JoVE* e50925 (2013) doi:10.3791/50925.

64. Berger, J., Sztal, T. & Currie, P. D. Quantification of birefringence readily measures the level of muscle damage in zebrafish. *Biochem. Biophys. Res. Commun.* **423**, 785–788 (2012).

65. Zahl, I. H., Samuelsen, O. & Kiessling, A. Anaesthesia of farmed fish: implications for welfare. *Fish Physiol. Biochem.* **38**, 201–218 (2012).

BIOGRAPHY OF THE AUTHOR

Sean Driscoll was born in Haverhill, Massachusetts in October 1996. Throughout his education, Sean has always enjoyed the study of science, no matter the field. In May of 2015, he earned his high school diploma from Haverhill High School and moved to the University of Maine where he will earn his B.S. in Zoology. During that time, he was a member of the UMaine Men's Swim Team and was the vice president of the Student Athlete Advisory Committee at UMaine, while serving in the America East Student Athlete Advisory Committee. He also completed his honors thesis with Dr. Michelle Goody in the Henry Lab and graduated with high honors. After graduation, Sean joined the Henry lab to complete his Master of Science in Zoology under Dr. Clarissa Henry. Upon completion of his degree, Sean will journey to the University of Massachusetts at Lowell where he will start his PhD in Applied Biology under Dr. Jennifer Fish. Sean is a candidate for the Master of Science in Zoology from University of Maine in May 2021.